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Cellular and Extracellular Protein Antigens of *Treponema pallidum* Synthesized During In Vitro Incubation of Freshly Extracted Organisms

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A new medium that permits radiolabeling of freshly extracted cells of *Treponema pallidum* with [^{35}S]methionine very efficiently has been devised. Although treponemes were not purified free of contaminating rabbit tissue, label was incorporated exclusively into treponemal protein in a linear manner for at least the first 16 h of in vitro incubation. Throughout this period, virtually a full complement of treponemal proteins was synthesized, based on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis comparison of the radiolabeled protein profile with the Coomassie blue-stained profile of gradient-purified treponemes. The radiolabeled protein profiles obtained with three pathogenic strains were very similar but not identical. Using solubilized treponemal extracts and a sensitive radioimmunoprecipitation procedure, we identified the protein antigens of *T. pallidum* that were recognized by immunoglobulin G antibodies in various rabbit and human syphilitic sera. A simple fractionation procedure has been used to separate soluble and membrane-bound treponemal proteins. A number of the membrane proteins are exposed on the cell surface, since intact radiolabeled treponemes bound antibodies directed against these proteins. In addition, a unique class of low-molecular-weight extracellular treponemal proteins has been identified. The cell surface-exposed proteins were among the earliest proteins recognized by immunoglobulin G antibodies after experimental infection of rabbits with *T. pallidum*.

Treponema pallidum is the etiological agent of syphilis, an infectious, sexually transmitted disease. Despite intensive study, very little is known about the virulence determinants of this organism or about the immunogenic determinants responsible for provoking a protective host immune response. It is also not known how this organism differs from the closely related pathogen, *T. pertenue*, the etiological agent of yaws, a nonsexually transmitted disease with a significantly different clinical course. Research on both these treponemal species has been seriously hampered by the inability to culture them in vitro for sustained periods. For experimental studies, these organisms are usually cultivated in rabbit testes, from which they can be extracted and purified in only small quantities. It is therefore difficult and expensive to obtain sufficient quantities of treponemes for meaningful analyses.

In an attempt to circumvent some of the problems associated with cultivation of *T. pallidum*, workers in several laboratories have sought to apply recombinant DNA techniques to the production of treponemal components (23, 28–30, 33, 34). In our laboratory, we have constructed several genomic libraries of *T. pallidum* DNA and identified a number of *Escherichia coli* clones expressing protein antigens specifically recognized by rabbit or human syphilitic and yaws sera, or both (28–30; L. V. Stamm, G. Bogosian, and P. J. Bassford, Jr., work in progress). It was our intention to focus our research efforts on just one or two of these clones. However, we quickly recognized that, based on the available information, it was very difficult to identify clones encoding *T. pallidum* protein antigens likely to be of particular interest from the standpoint of the pathogenicity or immunogenicity of the organism. For this reason, we found it necessary to develop our own criteria for the identification of such treponemal protein antigens and to undertake a detailed analysis of the native protein antigens of *T. pallidum* and, to a certain extent, *T. pertenue*.

In this study we have developed a new protocol for the in vitro radiolabeling of freshly extracted treponemes that has permitted us to easily identify treponemal proteins and protein antigens without extensive purification procedures. Using treponemes radiolabeled with [^{35}S]methionine, we have been able to identify a number of cell surface treponemal protein antigens, as well as a unique class of extracellular protein antigens. Since these are the proteins in direct contact with the tissues of the infected host, it is likely that at least some of these proteins have a major role in pathogenesis. In addition, we have found that certain of these proteins are among the earliest protein antigens recognized by the immunoglobulin G (IgG) response of infected rabbits, and so antibodies to these proteins may be an important component of the host immune response.

MATERIALS AND METHODS

Bacterial strains. The sources and cultivation of *T. pallidum* Nichols, *T. pallidum* street strain 14, *T. pertenue* Gauthier, and *T. phagedenis* biotype Reiter were previously described (30). *T. phagedenis* biotype Kazan, obtained from the laboratory of Robert Nauman, was cultivated at 37°C in Spirolate broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10% heat-inactivated normal rabbit serum (M.A. Bioproducts, Walkersville, Md.). *T. denticola* W and 11, were also obtained from Robert Nauman and were cultivated at 37°C in PPLO broth (BBL) supplemented with 10% heat-inactivated normal rabbit serum and 5 μg of cocarboxylase per ml (15). Treponemes were enumerated as previously described (21).

Extraction of virulent treponemes and radiolabeling with [^{35}S]methionine. Testes were aseptically removed from in-

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fected rabbits on the day of maximal orchitis. Testicular tissue was finely minced, and treponemes were extracted 5 to 8 times at 32°C in 15 ml of extraction medium for 15 min per extraction. The extraction medium was prepared as follows: 66 ml of distilled water; 5 ml of 10× M9 salts (20); 4 ml of 20% glucose; 2 ml of 0.1% thiamine; 1 ml of 12% glycerol; 1 ml of 5% sodium pyruvate; 7.5 ml of heat-inactivated normal rabbit serum; 10 ml of a freshly prepared and autoclaved solution of 1% cysteine and 0.5% thioglycolate; 4 ml of a stock solution of amino acids (30); 2 ml of 0.05 M leucine; 1 ml of a stock solution of threonine (5 mg/ml), proline (5 mg/ml), and arginine (10 mg/ml); 0;1 ml of 0.1 M $CaCl_2$; 0.1 ml of 0.1 M $MgCl_2$; 0.2 ml of 0.5% cocarboxylase; 0.1 ml of 1.5% dithiothreitol; and 0.1 ml of 1% sodium bisulfite. The medium components were gently mixed to avoid excessive aeration, the pH was adjusted to 7.5 with 1 N NaOH, and the medium was filter sterilized.

Extracted treponemes were centrifuged at 500 × *g* at room temperature for 10 min to pellet most of the rabbit tissue. Supernatant fluids containing the treponemes were pooled and centrifuged at 20,000 × *g* for 15 min. The pelleted treponemes were suspended in the extraction medium to a density of 3×10^8 to 6×10^8 cells per ml. Treponemes (1-ml volumes) were placed into sterile, glass screwcap vials. Cycloheximide (5 mg/ml stock; Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 100 μg/ml to inhibit protein synthesis by any contaminating cells of rabbit origin. Treponemal proteins were radiolabeled with 65 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml at 34°C either overnight (approximately 20 h) or for other times indicated. It is important to note that we have not made rigorous attempts to define the optimal concentration of each component in the extraction medium. However, we have found that the treponemes labeled very efficiently in this medium under the experimental conditions stated (see below).

To determine the incorporation of label into protein, a 0.1-ml sample was transferred from each vial to a tube containing 2.5 ml of cold 25% trichloroacetic acid (TCA)–2% methionine and placed on ice for 15 min. The TCA precipitate was trapped on glass microfibre filters (Whatman) and washed three times with 5 ml of 8% TCA–2% methionine. Filters were air dried and counted in 10 ml of scintillation fluid. The remaining sample in each vial was examined by dark-field microscopy and checked for bacterial contamination. Treponemes were transferred to 1.5-ml Eppendorf tubes and pelleted by centrifugation at room temperature for 3 min in a microfuge. The treponemes were washed twice with phosphate-buffered saline (PBS) and once with 10 mM Tris (pH 8.0), solubilized in 10 mM Tris (pH 7.5)–1 mM EDTA–1% sodium dodecyl sulfate (SDS), heated for 3 min in a boiling-water bath, and centrifuged for 10 min in a microfuge. Supernatants containing the radiolabeled treponemal proteins were removed and stored at −20°C.

Radiolabeling of cultivable treponemes with [^{35}S]methionine. The medium and conditions used for radiolabeling the cells of *T. phagedenis* biotypes Reiter and Kazan were previously described (30). Cells of *T. denticola* W and 11 were radiolabeled under similar conditions, except that the medium contained 5 μg of cocarboxylase per ml and the pH was 7.5.

Cell fractionation. Approximately 10^{10} cells of *T. pallidum* Nichols were radiolabeled overnight with [^{35}S]methionine as described above. At the end of the labeling period, treponemes were pelleted by centrifugation at 20,000 × *g* for 15 min at 4°C. The supernatant fluid, containing extracellular protein antigens synthesized during the overnight incubation period, was removed and processed as described below. The treponemes were washed twice in PBS and resuspended in 2 ml of 10 mM Tris (pH 8.0)–1 mM EDTA–2 mM phenylmethylsulfonyl fluoride. Treponemes were disrupted by sonication (six 5-s bursts with the Microtip at 30% power; model 300, Fisher Scientific Co., Pittsburgh, Pa.). DNase (Sigma) and 200 μl of 2.5 mM $MgCl_2$ were added to the sonic extract. After incubation at 4°C for 30 min, unlysed cells were removed by centrifugation in a microfuge for 15 min at 4°C. A 0.5-ml sample representative of the whole sonic extract was removed and processed as described below. Soluble and membrane proteins were separated by ultracentrifugation at 100,000 × *g* for 90 min at 4°C. The pellet (membrane fraction) was suspended in 1.5 ml of 10 mM Tris (pH 8.0)–0.5 M KCl by homogenization. Ultracentrifugation of the membrane and soluble fractions was repeated. The membrane fraction obtained was solubilized in 300 μl of 10 mM Tris (pH 7.5)–1 mM EDTA–1% SDS. Proteins in the soluble fraction and the whole sonic extract were precipitated by mixing with an equal volume of cold 10% TCA, incubation on ice for 15 min, and centrifugation in a microfuge for 2 min. The precipitates obtained were washed once with cold acetone. The soluble protein fraction was solubilized in 300 μl of 10 mM Tris (pH 7.5)–1 mM EDTA–1% SDS. The whole sonic extract proteins were solubilized in 100 μl of the same buffer. Each of the solubilized fractions was processed as described above for whole cells. The supernatant fluid containing extracellular protein antigens was twice clarified by centrifugation in a microfuge for 15 min at 4°C. The supernatant from the last centrifugation step was precipitated with an equal volume of cold 10% TCA. The precipitate was washed, solubilized, and processed as described above.

Detection of cell surface-exposed antigenic proteins. A modification of the whole-cell radioimmunoprecipitation method of Hansen et al. (12) was used to identify antibody-accessible proteins on the cell surface of *T. pallidum* Nichols. Briefly, [^{35}S]methionine-labeled intact cells were washed twice with PBS (35) and resuspended in the same buffer to approximately 1.4×10^9 treponemes per ml. Equal amounts (50 μl) of heat-inactivated normal rabbit serum, experimental rabbit syphilitic serum, or PBS were added to separate 1-ml portions of cells, and the mixtures were gently agitated at 4°C for 90 min. Next, treponemes were washed three times in PBS to remove unbound antibodies and other serum components. Antigen-antibody complexes were extracted in solubilization buffer (12) for 2 h at 37°C. The extracts were centrifuged for 15 min in a microfuge to remove insoluble material. A 200-μl portion of each extract was mixed with 100 μl of a slurry of protein A–Sepharose CL-4B (Sigma) and then incubated at 4°C for 60 min, again with gentle agitation. The protein A-antibody-antigen complexes were washed three times with solubilization buffer, twice with solubilization buffer without detergent, and once with 10 mM Tris (pH 8.0). Immunoprecipitates were extracted as previously described (30).

Sera. All rabbits used for the production of antisera were bled before infection or immunization to obtain control sera. Human syphilitic sera, experimental rabbit syphilitic sera, and rabbit anti-Reiter sera used in this study were described in detail previously (30).

Immunoprecipitation of radiolabeled treponemal proteins. Solubilized radiolabeled treponemal extracts (10 to 15 μl) or solubilized extracellular proteins (20 to 25 μl) were mixed with 500 μl of Triton buffer (30). A 5-μl volume of human serum or a 15-μl volume of rabbit serum was added, and the

mixture was incubated overnight at 4°C. On the following day, 100 μl of a slurry of protein A–Sepharose CL-4B (Sigma) was added. The mixture was incubated at 4°C for 90 min with gentle agitation and then pelleted and washed, and the immunoprecipitate was extracted as previously described (30).

SDS-PAGE and fluorography. The SDS-PAGE system used has been described previously (30). Radiolabeled cell extracts (mixed 1:2 with 2× sample buffer) and immunoprecipitates were electrophoresed on 15% acrylamide slab gels. The gels were stained, destained, and processed for fluorography as previously described (30). Molecular weights were determined on the basis of the positions of unlabeled known protein standards (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

Radiolabeling of *T. pallidum* with [^{35}S]methionine. In our hands, the procedure described by Baseman and coworkers (1–3) for the radiolabeling of cells of *T. pallidum* Nichols freshly extracted from infected rabbit testes did not work.

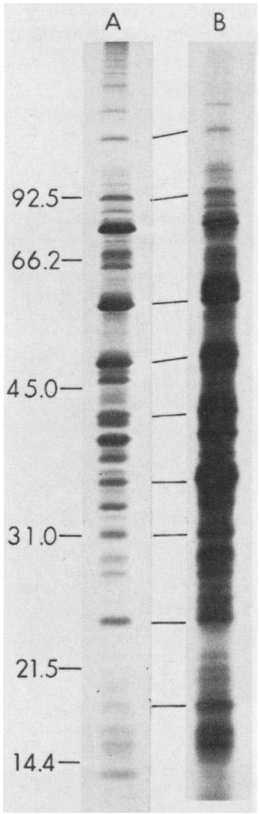

FIG. 1. SDS-PAGE comparison of the Coomassie blue-stained protein profile of gradient-purified *T. pallidum* Nichols with the protein profile obtained by fluorography of radiolabeled treponemal proteins. Lanes: (A) profile of treponemes extracted from infected rabbit testes, purified on Methocel-Hypaque gradients (1), solubilized in buffer containing 1% SDS, electrophoresed on a 15% polyacrylamide slab gel, and stained with Coomassie blue; (B) profile of freshly extracted treponemes radiolabeled with [^{35}S]methionine as described in the text, washed, solubilized, and electrophoresed on the same slab gel. This portion of the gel was prepared for fluorography. Numbers at left (×10^3) refer to the molecular weights of unlabeled known protein standards run on the same gel.

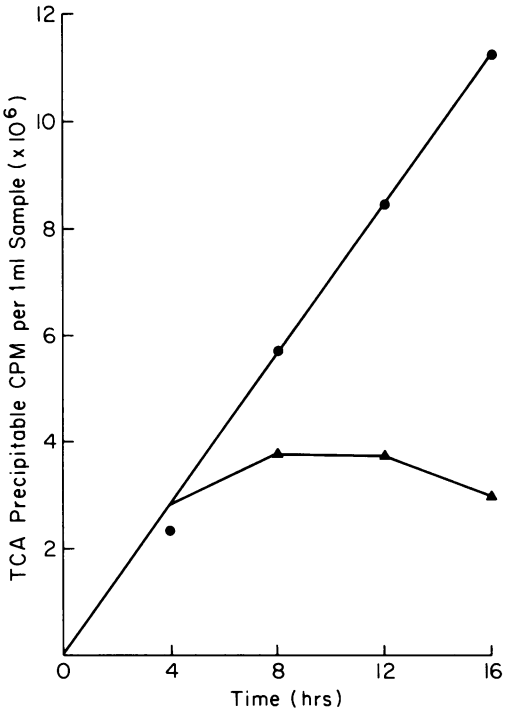

FIG. 2. Incorporation of [^{35}S]methionine into protein by freshly extracted *T. pallidum* Nichols. Treponemes freshly extracted from infected rabbit testes were resuspended in extraction medium containing cycloheximide to a density of 4 × 10^8 cells per ml. Symbols: ●, treponemes continuously incubated in the presence of [^{35}S]methionine for 4, 8, 12, or 16 h; ▲, treponemes incubated for 4 h with [^{35}S]methionine after preincubation in extraction medium without label for 4, 8, or 12 h. At the end of each labeling period, a 0.1-ml sample was precipitated with TCA and counted, as described in the text.

However, with the procedure described above, freshly extracted treponemes could be efficiently radiolabeled with [^{35}S]methionine. Treponemes obtained after an overnight incubation in the labeling medium were analyzed by one-dimensional SDS-PAGE and fluorography. This SDS-PAGE protein profile was compared with that obtained by Coomassie blue staining of proteins prepared from gradient-purified treponemes (Fig. 1). Although there were detectable differences in the intensity of some individual proteins, the two profiles were largely identical. It therefore appeared that virtually a full complement of treponemal proteins were synthesized and radiolabeled under the conditions used. Our evidence indicated that label was incorporated only into proteins of treponemal origin. The labeling medium included cycloheximide to inhibit eucaryotic protein synthesis. We found that label was not incorporated into protein in the presence of tetracycline (4 μg/ml) or chloramphenicol (20 μg/ml) (data not shown). *T. pallidum* is known to be sensitive to both of these antibiotics (14).

With our radiolabeling procedure, [^{35}S]methionine was incorporated into TCA-precipitable material in a linear fashion for at least 16 h. In the experiment shown in Fig. 2, we obtained nearly 1.2 × 10^7 TCA-precipitable cpm of [^{35}S]methionine incorporated into approximately 4 × 10^8 treponemes over a 16-h period. Treponemes radiolabeled for various periods were analyzed by SDS-PAGE and fluorography (Fig. 3). With a few minor variations, each of the protein bands increased in intensity in proportion to the time that the treponemes were incubated in the presence of

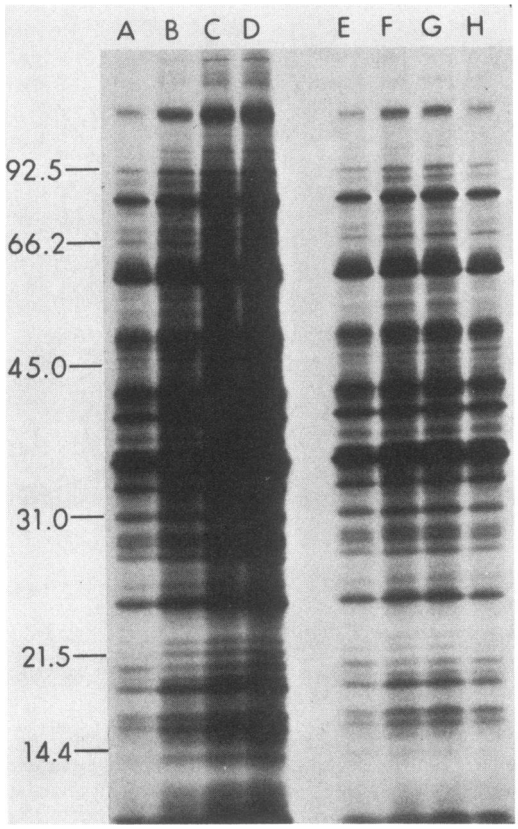

FIG. 3. Analysis of radiolabeled proteins of *T. pallidum* Nichols by SDS-PAGE and fluorography. Lanes: (A) through (D) profiles of treponemes continuously labeled with [^{35}S]methionine for 4, 8, 12 or 16 h, washed, solubilized, and analyzed by SDS-PAGE and fluorography; (E) through (H) treponemes labeled for 4 h with [^{35}S]methionine after preincubation in the extraction medium without label for 0, 4, 8 or 12 h, washed, solubilized, and analyzed by SDS-PAGE and fluorography. Note that these samples came from the experiment described in Fig. 2. See text for additional experimental details.

[^{35}S]methionine. This indicated that, despite the fact that treponemes were not multiplying under these conditions, there was little turnover of treponemal proteins. In a separate experiment, treponemes were extracted into labeling medium without [^{35}S]methionine. Treponemes then were pulse labeled with [^{35}S]methionine for 4-h periods beginning at 0, 4, 8, and 12 h postextraction. Treponemes incorporated an approximately equal amount of label into TCA-precipitable material during each of these four intervals (Fig. 2). The proteins labeled during each of these intervals were analyzed (Fig. 3). It was apparent that treponemes were continuing to synthesize a seemingly full complement of proteins throughout the 16-h incubation period.

SDS-PAGE protein profiles of different treponemal strains. Using the same protocol, [^{35}S]methionine was efficiently incorporated into treponemes freshly extracted from rabbits infected with *T. pallidum* street strain 14 and *T. pertenue* Gauthier. This permitted us to directly compare the protein profiles of these two pathogenic treponemal strains with that of *T. pallidum* Nichols (Fig. 4). These three treponemal strains exhibited nearly identical protein profiles. The minor differences easily discerned among the three strains are indicated. These protein profiles were compared with those exhibited by various cultivable treponemal strains. Although the proteins of two different biotypes of *T. phagedenis* were very similar, as were the proteins of two different strains of *T. denticola*, there was no obvious relationship between the protein profiles displayed by these three groups of treponemes.

Protein antigens of pathogenic treponemes as determined by radioimmunoprecipitation. To determine the treponemal protein antigens recognized by IgG antibodies present in various rabbit sera, radiolabeled treponemal antigen extracts were prepared and the protein antigens were precipitated and analyzed by SDS-PAGE and fluorography. Normal rabbit serum failed to precipitate *T. pallidum* Nichols proteins (Fig. 5). High-titer experimental rabbit syphilitic serum (ERSS) obtained from individual animals infected with any one of three pathogenic treponemes precipitated a large number of treponemal proteins. The presence in these sera of IgG antibodies that recognized most of the proteins that could be discerned in the respective antigen extracts was apparent. Similar results were obtained with protein antigens of *T. pallidum* street strain 14 and *T. pertenue* (not shown). For *T. pallidum* Nichols, there were minor differences in the protein antigens recognized by sera from rabbits infected with different pathogenic strains. These differences probably reflected the minor differences we discerned in the respective protein profiles. For example, there was a protein with a molecular weight of approximately 35,000 (35K

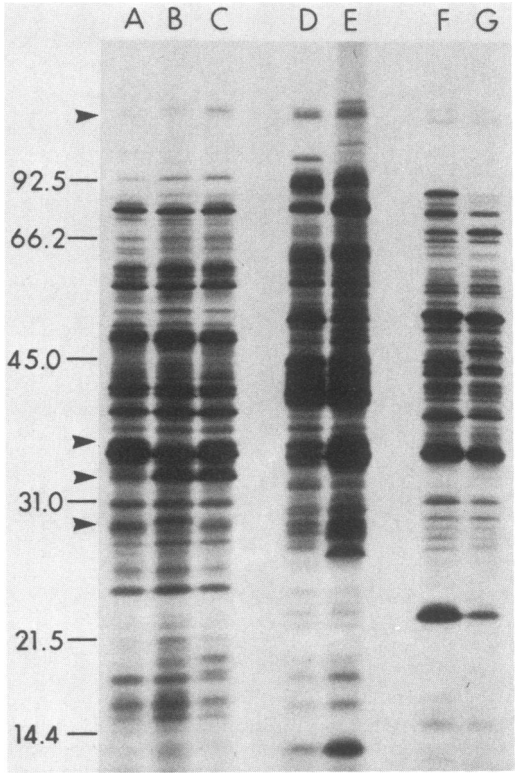

FIG. 4. Comparison of radiolabeled protein profiles of various pathogenic and cultivable treponemal strains by SDS-PAGE and fluorography. Treponemes were radiolabeled with [^{35}S]methionine, washed, solubilized, and analyzed by SDS-PAGE and fluorography, as described in the text. Lanes: (A) *T. pallidum* Nichols; (B) *T. pallidum* street strain 14; (C) *T. pertenue* Gauthier; (D) *T. phagedenis* biotype Reiter; (E) *T. phagedenis* biotype Kazan; (F) *T. denticola* W; and (G) *T. denticola* 11. Minor differences in the protein profiles of the three pathogenic treponemal strains are indicated by arrows at left.

protein) in the Nichols antigen extract (Fig. 5, arrow) that was efficiently precipitated by sera from rabbits infected with either *T. pallidum* Nichols or *T. pertenue* but was only very inefficiently precipitated by serum from a rabbit infected with *T. pallidum* street strain 14. Since the corresponding 35K protein was missing in the protein profile of street strain 14 (Fig. 4), this result was not surprising and serves to illustrate the specificity of the radioimmunoprecipitation procedure we used. In addition, note that rabbit anti-Reiter serum precipitated several *T. pallidum* Nichols protein antigens (Fig. 5). Previous studies have demonstrated a small degree of cross-reactivity between the protein antigens of cultivable and noncultivable treponemal strains (11, 16, 30, 31). This was not investigated further in this study.

The development of an IgG antibody response to the protein antigens of *T. pallidum* Nichols in rabbits experimentally infected with this strain was investigated. As previously reported (30), three rabbits were intratesticularly infected with *T. pallidum* on day 0. Each rabbit was monitored for the development of an orchitis, and blood was drawn for serum on various days postinfection. Rabbits were not immunosuppressed with cortisone. The sera obtained from these rabbits preinfection and postinfection were tested for their ability to precipitate treponemal proteins. The protein antigens identified by this analysis were quite similar for each of the infected rabbits; the results for just one rabbit are shown in Fig. 6. IgG antibodies directed against *T. pallidum* Nichols proteins were first detected in sera taken from these animals on day 11 postinfection. This corresponded with the development of peak orchitis in each animal. At this juncture, only a very limited number of proteins were precipitated from the antigen extract and only a single protein with a molecular weight of approximately 40,000 was efficiently recognized. Sera taken at later times postinfection recognized a greater number of protein antigens, and the efficiency with which various proteins were precipitated also increased with time. IgG antibodies directed against most treponemal proteins were detected by around day 35 postinfection; little loss in reactivity was noted in serum samples taken as late as day 490 postinfection. It is of interest to note that there was a definite decrease in the efficiency with which the 40K protein mentioned above was precipitated by serum taken at day 14 postinfection. The efficiency in precipitation of this protein once again increased with serum taken on day 17 postinfection. This was observed with serum samples from each of the three infected rabbits and corresponded to the period when treponemes were being cleared from the testes.

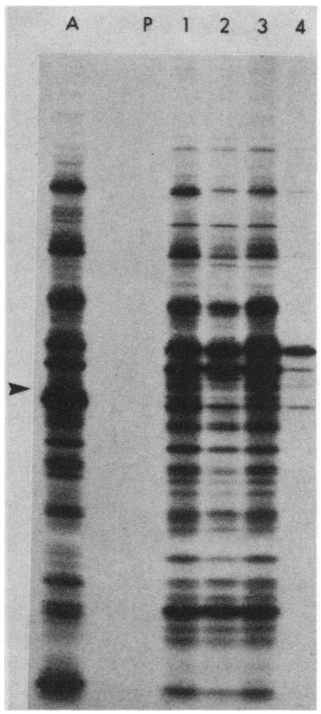

FIG. 5. Radioimmunoprecipitation of *T. pallidum* Nichols protein antigens with various rabbit sera. Immunoprecipitations with a solubilized, radiolabeled treponemal extract, rabbit serum, and protein A-Sepharose were performed as described in the text. Precipitates were analyzed by SDS-PAGE and fluorography. Lanes: (A) whole cell antigen extract; precipitates obtained with (P) control rabbit serum; (1) high-titer serum from rabbits experimentally infected with *T. pallidum* Nichols; (2) high-titer serum from rabbits infected with *T. pallidum* street strain 14; (3) high-titer serum from rabbits infected with *T. pertenue*; and (4) serum from a rabbit immunized with *T. phagedenis* biotype Reiter. The 35K protein mentioned in the text is indicated by an arrow.

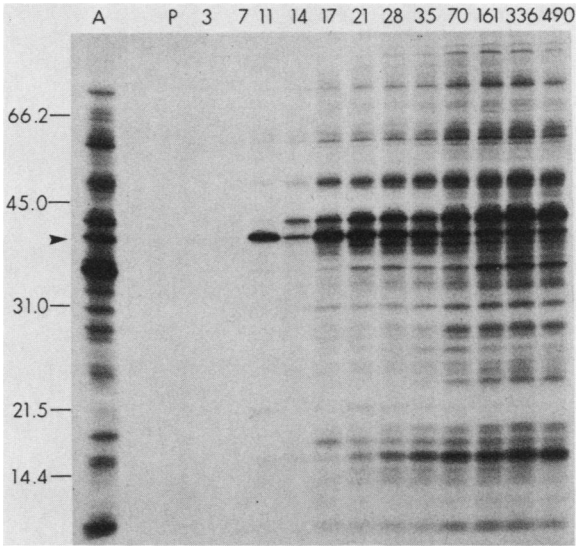

FIG. 6. Development of an IgG antibody response to *T. pallidum* Nichols protein antigens in an experimentally infected rabbit. Immunoprecipitations were performed with solubilized, radiolabeled treponemal extracts, using serum samples obtained from a rabbit at various times postinfection. Precipitates were analyzed by SDS-PAGE and fluorography. Numbers above each lane are the number of days postinfection that the serum sample was obtained. Lanes: (A) solubilized *T. pallidum* Nichols extract; (P) precipitate obtained with serum from a rabbit taken preinfection. The 40K protein mentioned in the text is indicated by an arrow.

We also investigated the ability of various human sera to precipitate *T. pallidum* Nichols protein antigens (data not presented). We found that individual normal human sera recognized a small number of proteins with varying efficiency. Primary human syphilitic sera (HSS) recognized additional proteins not precipitated by normal human sera. The protein that appeared to be recognized most strongly by each of the primary HSS tested was the same 40K protein most efficiently precipitated by serum samples taken from rabbits at early times postinfection. Reactivity of secondary and latent HSS with treponemal protein antigens was significantly greater than that obtained with primary HSS. Although some variability was exhibited by individual sera, in

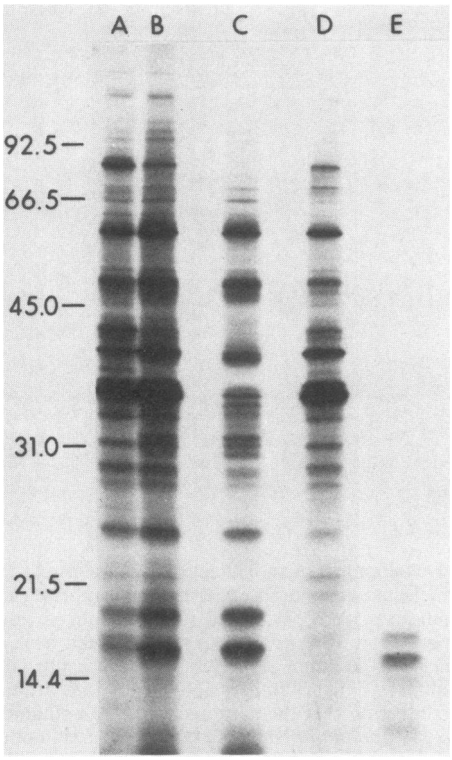

FIG. 7. Fractionation of [^{35}S]methionine-labeled *T. pallidum* Nichols proteins. Soluble, membrane-bound, and extracellular proteins of radiolabeled treponemes were prepared as described in the text and analyzed by SDS-PAGE and fluorography. Lanes: (A) whole cells solubilized in buffer containing 1% SDS; (B) whole sonic extract; (C) soluble protein fraction; (D) membrane protein fraction; (E) extracellular protein fraction. See text for additional experimental details.

general the results were similar to those obtained with high-titer ERSS. We found a considerable decrease in the reactivity of tertiary HSS with treponemal protein antigens. A large number of proteins were still recognized by these sera, but the efficiency with which individual proteins were precipitated in most cases was greatly reduced.

Soluble and membrane-bound proteins of *T. pallidum*. Cells of *T. pallidum* Nichols were radiolabeled and disrupted by sonication, and the membrane fraction was separated from soluble proteins by ultracentrifugation. The proteins present in the total sonic extract and each treponemal fraction were analyzed (Fig. 7). The protein profile exhibited by the total sonic extract was nearly identical to that of whole cells solubilized in SDS. Although some differences were discerned, probably as a result of a proteolytic activity uncovered when treponemes were disrupted under nondenaturing conditions, most of the major protein bands were not affected. Not surprisingly, the soluble and membrane fractions exhibited unique protein profiles. Several proteins appeared to reside in both fractions. These may be comigrating species or may represent peripheral membrane proteins that were partially removed from the membrane surface when the cells were disrupted. A careful comparison of Fig. 6 and 7 revealed that most of the major protein antigens recognized by serum samples taken from rabbits at early times postinfection were found in the membrane fraction.

Cell surface proteins of *T. pallidum*. Hansen et al. (12) described a method that permits one to identify cell surface proteins by using the appropriate antiserum and whole, intact cells that have been radiolabeled. We have used this "whole cell radioimmunoprecipitation" procedure to identify the cell surface protein antigens of *T. pallidum*. Radiolabeled treponemes were washed and then incubated for 1 h with either control rabbit serum or high-titer serum from a *T. pallidum* Nichols-infected rabbit. The unbound antibodies were removed by extensive washing, the treponemes were solubilized, and antigen-antibody complexes were precipitated with *Staphylococcus aureus* protein A and analyzed (Fig. 8). Control rabbit serum did not recognize any treponemal proteins. ERSS recognized a number of distinct proteins. Each of these proteins corresponded to a specific membrane protein that was identified by the simple fractionation technique described above. Particularly prominent among the protein antigens identified by this procedure were proteins with approximate molecular weights of 16,000, 42,000, and 48,000 (indicated by arrows). The 40K protein mentioned above was also clearly a cell surface protein by this criterion; however, this protein did not appear to be solubilized well in the Hansen buffer. Sera from rabbits

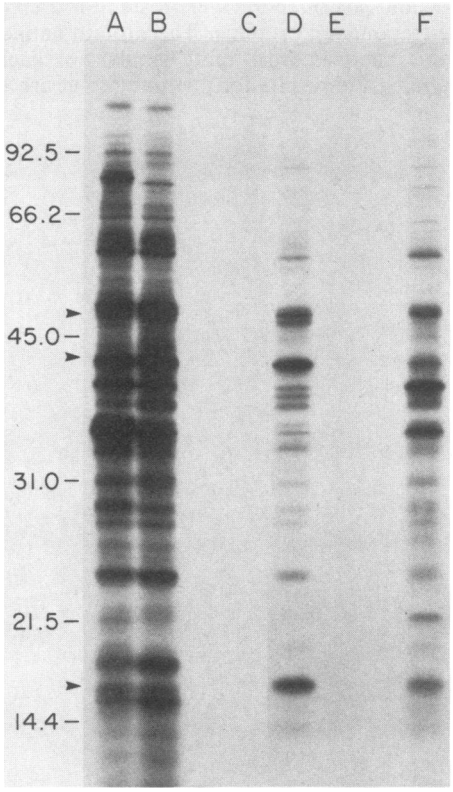

FIG. 8. Radioimmunoprecipitation of *T. pallidum* Nichols cell surface-exposed protein antigens. Intact radiolabeled treponemes were incubated with control rabbit serum, serum from a rabbit experimentally infected with *T. pallidum* Nichols, or buffer. Cells were washed free of unbound antibody. Antigen-antibody complexes were extracted with solubilization buffer and precipitated with protein A-Sepharose (see the text for details). Precipitates were analyzed by SDS-PAGE and fluorography. Lanes: (A) [^{35}S]methionine-labeled treponemes solubilized in buffer containing 1% SDS; (B) treponemes solubilized in buffer described by Hansen et al. (12); (C) precipitate obtained with control rabbit serum; (D) precipitate obtained with ERSS; (E) precipitate obtained with buffer alone; (F) membrane protein antigens recognized by ERSS (Fig. 7). Arrows at left indicate (from top) the 48K, 42K, and 16K surface proteins that are mentioned in the text.

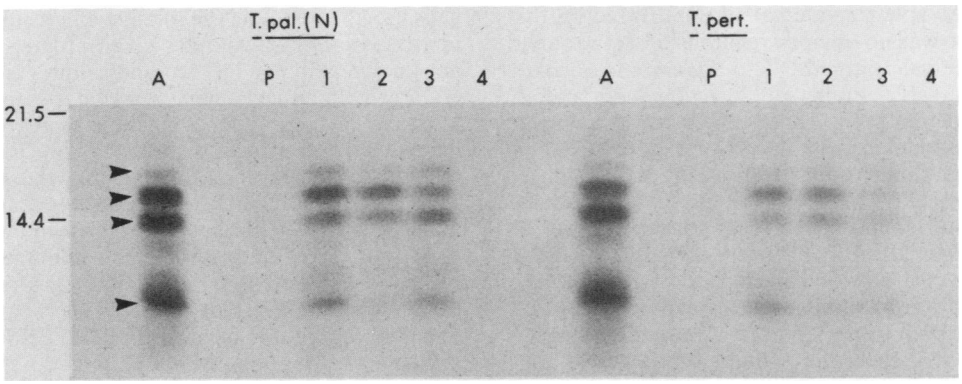

FIG. 9. Radioimmunoprecipitation of extracellular protein antigens of *T. pallidum* Nichols and *T. pertenue* with various rabbit sera. Extracellular proteins present in cell-free supernatants of radiolabeled treponemes were concentrated by TCA precipitation. Solubilized extracts were prepared and immunoprecipitations were performed with rabbit sera and protein A-Sepharose.The precipitates were analyzed by SDS-PAGE and fluorography. Lanes: (A) extracellular treponemal protein extract; precipitates obtained with (P) control rabbit serum; (1) serum from rabbits experimentally infected with *T. pallidum* Nichols; (2) serum from rabbits experimentally infected with *T. pallidum* street strain 14; (3) serum from rabbits experimentally infected with *T. pertenue*; and (4) rabbit anti-Reiter serum. Arrows at left indicate the protein antigens of (from top) the 17K, 15.5K, 14K, and 10.5K that are mentioned in the text. Note that only the relevant portion of the gel is shown.

infected with *T. pallidum* street strain 14 or *T. pertenue* yielded identical results (data not presented).

Extracellular protein antigens of *T. pallidum* and *T. pertenue*. We considered the possibility that a specific class of proteins was synthesized and secreted by *T. pallidum* and *T. pertenue* strains. Treponemes radiolabeled by overnight incubation in the presence of [^{35}S]methionine were removed from the labeling medium by centrifugation. Total supernatant proteins were precipitated with ice-cold TCA. To test for the presence of radiolabeled protein species, the TCA precipitate was solubilized in SDS sample buffer and analyzed by SDS-PAGE and fluorography. Four low-molecular-weight proteins (approximately 10,500, 14,000, 15,500, and 17,000) were easily discerned in a fluorograph of a 15% acrylamide gel (Fig. 9). The gel was nearly devoid of radiolabeled, higher-molecular-weight proteins (Fig. 7). In addition, since proteins with a similar migration pattern were not discerned in either the membrane or the soluble fraction derived from whole, washed treponemes, it appeared that these low-molecular-weight proteins were not released from treponemes by cell lysis but rather that they represent a unique class of extracellular proteins. These extracellular proteins were not synthesized when protein synthesis was inhibited with chloramphenicol, further demonstrating that these proteins were of treponemal origin. The extracellular proteins were specifically precipitated by serum from rabbits experimentally infected with either *T. pallidum* or *T. pertenue* strains (Fig. 9). Note that these proteins clearly were not precipitated by rabbit anti-Reiter serum. A similar class of extracellular proteins could not be identified in the culture supernatant of Reiter treponeme (data not shown). Finally, we found that the majority of sera taken from human syphilis patients beyond the primary stage of disease precipitated several or all of these proteins (data not presented). Thus, it seems clear that these extracellular treponemal protein antigens are synthesized during human infection and elicit an IgG antibody response.

DISCUSSION

We have used an essentially new procedure for radiolabeling the proteins of pathogenic treponemal strains freshly extracted from infected rabbit testes. We found that incorporation of [^{35}S]methionine into treponemal protein was linear for at least the first 16 h of in vitro incubation in the labeling medium. Although treponemes did not multiply under these conditions, it would seem that a very nearly complete complement of treponemal proteins was synthesized throughout this 16-h period. In addition, there was no indication of turnover of radiolabeled treponemal proteins in vitro.

We are not the first to describe the radiolabeling of *T. pallidum* and *T. pertenue* strains in vitro. It is difficult to make a direct comparison of the different methods that have been used. Baseman and coworkers (1–3, 5–7, 32) have radiolabeled treponemes with ^{3}H- and ^{14}C-labeled amino acids as well as with [^{35}S]methionine, and we have drawn upon their results in devising our radiolabeling procedure. Baseman and Hayes (6) found that incorporation of label was linear for at least 24 h in their system, but the efficiency of incorporation of isotopically labeled amino acids into protein was considerably less than that reported here. We also obtained approximately 10-fold higher incorporation of [^{35}S]methionine into treponemal proteins than that recently reported by Thornburg and Baseman (32). The two procedures appear to radiolabel the same *T. pallidum* Nichols proteins. R. Thornburg provided us an aliquot of treponemes he had radiolabeled. We directly compared the protein profiles of treponemes radiolabeled by the two methods and found them to be virtually identical (data not presented). A major difference in the two procedures is that we did not gradient purify the treponemes before their incubation in the labeling medium. We found that [^{35}S]methionine was incorporated only into proteins of treponemal origin. Thus, our preparation was at least "isotopically pure." We feel that it is a major advantage to subject treponemes to a minimum of stress and to place them into the radiolabeling medium as soon as possible after extraction. Moskophidis and Müller (22) also did not purify treponemes before radiolabeling them. The efficiency of labeling they obtained with either [^{35}S]methionine or ^{14}C-amino acids was not reported. These workers did not include serum in their labeling medium, and a number of discrepancies were noted between their SDS-PAGE profiles of treponemal proteins obtained by Coomassie blue staining and autoradiography.

We compared the protein profiles of a number of non-

cultivable and cultivable treponemal strains. Based on this one criterion, there was no obvious relationship between the pathogenic treponemal strains of *T. pallidum* and *T. pertenue* and the cultivable strains of *T. phagedenis* and *T. denticola* that were analyzed. These organisms also appear to be genetically unrelated (19). As previously reported by others (4, 32), we found that the protein profiles of *T. pallidum* Nichols and *T. pertenue* Gauthier were nearly identical. This result supports the recent designation of *T. pertenue* as a subspecies of *T. pallidum* (27). Interestingly, the protein profile of a more recent isolate of *T. pallidum*, designated street strain 14, exhibited several differences that distinguish it from both of the other two strains.

We have found that high-titer rabbit sera and HSS contained IgG antibodies that specifically precipitated the great majority of radiolabeled treponemal proteins present in the solubilized extract. This was a larger number of protein antigens than was detected by other investigators using a different radioimmunoprecipitation procedure (2, 3, 5, 22, 32) and may reflect the sensitivity of our procedure, different gel conditions, and the fact that we obtained more efficient incorporation of label into treponemal proteins. We also detected a larger number of protein antigens than those identified by Western blotting techniques (4, 9, 10, 32). In general, we have found that radioimmunoprecipitation is a somewhat more sensitive technique. The fact that high-titer sera precipitated the great majority of treponemal proteins should not be particularly surprising, since presumably an animal or a human should be exposed to each of these protein antigens during the course of infection. Injection of suspensions of whole cells into an animal should result in the production of antibodies directed against all immunogenic materials present in the cells (8).

Another result that was not unexpected was the finding of a very high degree of cross-reactivity between the protein antigens of different pathogenic treponemes. Others (4, 32) had previously noted that the antigenic profiles of *T. pallidum* Nichols and *T. pertenue* were nearly identical. Using Western blotting, Baker-Zander and Lukehart (4) had discerned two differences between these two strains that we were unable to detect in this study. We have expanded upon these earlier observations by including *T. pallidum* street strain 14. High-titer serum from a rabbit infected with street strain 14 precipitated most *T. pallidum* Nichols protein antigens. The several exceptions encountered could be explained by differences in the respective protein profiles. The significance of these differences remains to be established. However, findings such as these emphasize the need to survey additional isolates of *T. pallidum* and *T. pertenue* and indicate that it may be very difficult to identify a specific protein antigen whose presence or absence can serve to differentiate between strains isolated from syphilis and yaws patients.

Major goals of our research are to identify the protein antigens of *T. pallidum* and *T. pertenue* that have important roles in the pathogenesis of disease or in generating a protective host immune response, or both. In this study we have initiated efforts at identifying such key protein antigens. The criteria that we are using and the results that we have obtained so far can be summarized as follows.

(i) IgG antibodies directed against *T. pallidum* protein antigens were first detected in serum samples obtained from rabbits on day 11 postinfection, corresponding to the development of peak orchitis in each animal. This is in close agreement with results of other studies (3, 10). We found that only a distinct subset of treponemal proteins were recognized at this point. The total number of protein antigens precipitated, and the relative efficiency of precipitation, increased in serum samples taken at later time points. It is not known whether IgG antibodies directed against *T. pallidum* protein antigens have a major role in the clearance of treponemes from infected rabbit testes during the days immediately after peak orchitis (17, 26). However, if such a role can be established, presumably the subset of treponemal protein antigens recognized by IgG antibodies present in serum samples obtained during this period must include important target antigens for the immune system. In this regard, we found that a specific, apparently major 40K treponemal protein was efficiently precipitated by ERSS obtained on the day of peak orchitis. ERSS taken from the same animal just 3 days later exhibited a markedly reduced ability to precipitate the same protein. A likely explanation for this observation is that 3 days after peak orchitis, a significant proportion of the IgG antibodies directed against this 40K protein have been removed from the circulation by antigens present in the infected animal. Also, as previously observed by Hanff et al. (9, 10), we found that the IgG response of humans to treponemal infection, through at least the early latent stage of disease, was markedly similar to that of infected rabbits. We did detect a decrease in the ability of late HSS to recognize treponemal proteins. However, unlike Hanff et al. (10), we were unable to identify specific protein antigens against which IgG antibodies were specifically missing in these sera.

(ii) Protein antigens situated on the treponemal cell surface are of interest for several reasons. First, these are the only cellular proteins that have direct access to the infected host. Such proteins may have particularly important roles in the pathogenesis of disease, e.g., mediating the attachment of treponemes to host tissues, damaging the host in such a manner as to permit spread of the organism, and allowing the organism to evade the host immune response. Second, a protective host immune response presumably must be targeted against antigenic determinants exposed on the cell surface. We used a simple fractionation procedure to separate the soluble and membrane-bound proteins of *T. pallidum*. We found that a fairly large subset of the membrane protein antigens appeared to be exposed on the treponemal cell surface, on the basis that intact cells bound antibodies directed against these proteins. Most of the major membrane proteins appeared to be surface exposed, including the 40K protein discussed above. However, it was clear that three of the membrane proteins (the 16K, 42K, and 48K proteins) were more efficiently precipitated by this procedure than were most other proteins. The significance of this result is unknown, although it is possible that access of antibody to some surface proteins is hindered by a surface layer of avidly bound proteins of host origin (1). Further studies are required to determine which of these protein antigens are actually surface exposed in infected rabbits or humans, since evidence suggests that the treponemal cell surface changes in its properties during in vitro incubation (13, 24, 25). However, it is interesting that, in infected rabbits, most of the protein antigens recognized early in infection, including the 16K, 42K, and 48K surface-exposed proteins, fractionated as membrane proteins. Using a surface-labeling technique, Alderete and Baseman (2) previously identified a number of *T. pallidum* surface proteins, certain of which have been implicated as attachment ligands (5). Owing to the different experimental conditions, it is difficult to correlate their results with ours, although we would certainly suspect that the different methods would identify a similar subset of treponemal proteins. Also, we suspect that the 48K surface protein we have identified is the same surface protein

recently identified by Marchitto et al. (18) with monoclonal antibodies.

(iii) We have identified a class of unique, low-molecular-weight protein antigens that appeared in the extracellular medium during in vitro radiolabeling of *T. pallidum* and *T. pertenue* strains. To our knowledge, this is the first indication that the pathogenic treponemes synthesize a class of extracellular proteins, and it is a result that we find quite intriguing. The properties that make these organisms successful human pathogens have not been elucidated. However, we feel that anything that these organisms secrete into their external environment, where it has the opportunity to interact with the infected host, must be considered a potential virulence determinant.

Obviously, this study leaves a great many questions unanswered. However, we believe that we have identified a number of cell surface and extracellular proteins of *T. pallidum* that must be considered potentially interesting. Efforts are now under way in our laboratory to screen genomic libraries of *T. pallidum* Nichols and *T. pallidum* street strain 14 DNA for *E. coli* clones expressing these proteins. In this way we hope to learn more about these proteins and their role in treponemal infections.

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